CAAP Quarterly Report, FY '25 Q1
Dec. 31, 2024

Project Name: Rhamnolipid: a Bio-based, Ecologically Friendly, Corrosion Inhibitor and SRB Biocide for Crude Pipelines
Contract Number: 693JK32350001CAAP
Prime University: University of Akron
Prepared By: Scott Lillard, rsl@uakron.edu, 330-972-7463
Reporting Period: Oct. 1, 2024 thru Dec. 30, 2024.

Project Activities for Reporting Period:

Students / Hiring.

New graduate students: Jan. 2025, Elaheh Mozayan, M.S. Biochem., University of Kashan, Iran; Jan. 2025 or May 2025: Kingsford Duah Agyemang, B.S. Petroleum Eng., Kwame Univ.
Current graduate students: Uddipta Mondal, B.S. Chem. Eng., BUET and Tijani Abdul-Gafaru B.S. Petroleum Eng., Kwame Univ.
Current undergraduate students: Ellie Zimmerer (Chem. Eng.), Rosemary Sterling (Chem. Eng.), Callie Lewis, (Chem. Eng.), Lily Clemente (Chem. Eng.).
Previous undergraduate students: Joseph Botzman (Corr. Eng) and Mikey Markov (Corr. Eng.).

Milestones

Table 1 lists the milestones for this project and their approximate status. As seen in this table, we are nearing completion or our produced water experiments, approximately 75% complete. These were our initial "proof of concept experiments" which were very successful showing that RhL is an effective inhibitor in simulated produced water. Project emphasis is now focusing on %IE in crude simulants and efficacy of RhL as an SRB biocide/MIC inhibitor.

Table 1. List of milestones (from proposal) and approximate status. Note PW stands for produced water.

	Status	Sched. Begin	Sched. End
1. *RhL Fermentation*	ongoing	10/9/23	5/30/26
2. *Corrosion, %IE and Mechanism*	30% complete	10/9/23	5/25/26
Purchase consumables, cell mods.	complete	10/9/23	12/11/23
Produced Water Exp,	75% complete	12/18/23	8/31/25
Crude Surrogate Exp.	on schedule	1/20/25	9/1/25
Actual Crude Exp.		9/22/25	5/25/26
3. *SRB-MIC*		9/1/24	6/1/26
Flow System Mods	on schedule	1/06/25	5/11/25
PW-SRB CorrRate (static)	30% complete	9/1/24	5/1/25
PW-SRB Attachment, CorrRate (flow)		5/12/25	5/12/26
4. *Di-RhL vs. Mono-RhL*		9/2/25	6/1/26
Separation of Di and Mono		9/2/25	12/31/25
CorrRate IE		1/1/26	5/30/26

Milestone 1, RhL Fermentation

In this quarter the student on this milestone made two new batches of fermentation for RhL production, learned how to measure RhL concentrations in fermentation samples, and established the relationship between cell dry weight (CDW) concentration and intracellular protein concentration in fermentation samples. These are described below in more detail.

New batches of RhL fermentation were made using the basic fermentation procedure as described in a previous quarterly report. In the previous fermentation, we used similar feed tubes for all supplement lines. However, in this run we used clog-resistant tubes for oil and viscous antifoam supply and calibrated them before operation. For seed culture preparation, we covered the shake flask with cotton-sandwiching cheesecloth (instead of aluminum foil) to improve oxygen transfer and minimize the chance of contamination. The seed culture was grown for 16 hours (instead of 20 hours) and inoculated to the fermentor, to ensure the cells were active (in the late exponential-growth phase). The fermentor was agitated at 800 rpm. Temperature was controlled at 32 °C. pH control set-point was 5.7. DO control set-point was 10%, with automatic on/off supply of pure oxygen. The O_2 flow rate was manually adjusted periodically to ensure that DO never drop below 5% air saturation or rise above 100%. The initial medium had 50 g/L soybean oil (previously 100 g/L which resulted in the presence of excess oil in the fermentor). Additional oil and nitrogen nutrient were slowly and separately added continuously according to designed adding schemes. The fermentation was run for 170 h. Results of CDW and RhL concentration profiles are shown in **Figure 1**. RhL production reached 16 ± 2 g/L but was too slow before 120 h. Also, the cell concentration reached only about 16 g/L, much lower than the about 30 g/L according to the limiting-nutrient design. These were attributed to insufficient soybean oil feeding, which was corrected (too late) at 120 h. DO overshooting also occurred occasionally in the early stage, which could stimulate production of other (non-RhL) metabolic products. These factors were taken into account in the next batch of fermentation to improve RhL production.

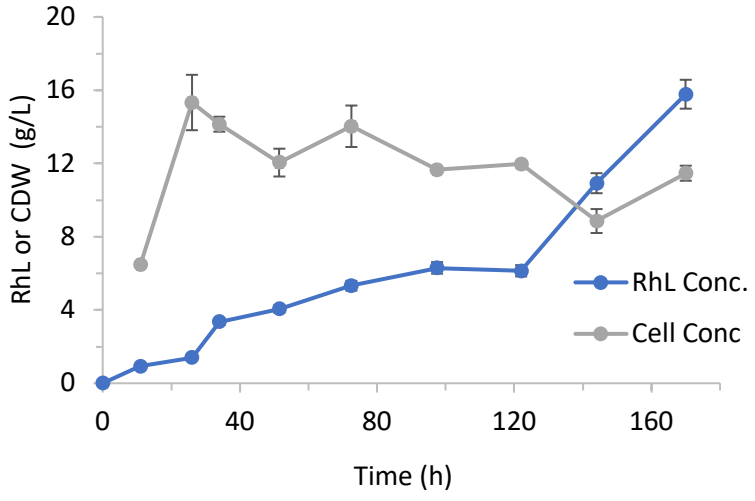

Figure 1 Rhamnolipid and cell-dry-weight profiles in a new batch of fermentation

In the previous fermentation, we encountered several operational issues: oversized pump-tubing diameters used for continuous addition of oil and N-source, respectively; insufficient soybean oil feeding; too much foaming; acid and base for pH control being added on top of foam layer, with

delayed reach to actual fermentation broth and harmful effects to cells in the foam layer; and occasional DO overshooting. These issues caused the lower-than-expected cell and RhL concentrations achieved. In the second batch of fermentation, we used smaller pump-tubing in the supply line for suitable flow rates and measured and tracked the added weights of N-source and oil along the fermentation. The antifoam agent tended to phase-separate after autoclaving. To avoid this, we added a magnetic stir-bar to the antifoam flask to keep it continuously mixed, for ensuring addition of a homogeneous mixture of antifoam agent. The DO probe was calibrated at 100% with saturated air in the initial medium with agitation at 800 rpm. Acid and base addition lines were extended into the fermentation broth, so the added acid and base would not stay on top of the foam layer. Also, to minimize the dilution effect due to the addition of large volumes of acid and base, 4 N HCl and 2 N NaOH were used in this fermentation for pH control (instead of the 1 N HCl and 1 N NaOH used previously). Further, while keeping the initial soybean oil concentration at 50 g/L, we started the supplemental soybean oil addition from the beginning at a 1.5 g/h rate to avoid the problem of C-source limitation encountered in the previous fermentation. The DO overshooting was controlled by adjusting the oxygen flow rate and changing the response time of opening and closing of the oxygen feed valve by selecting optimal p-gain and i-gain values. The fermentation is ongoing. We will report the results in the next quarterly report.

To quantify RhL concentration, fermentation samples were diluted 10 times with a $NaHCO_3$ solution and centrifuged at 5,900 g for 10 min. The supernatants collected were added with 4 N HCl to drop the pH to about 2, to protonate and precipitate RhL. We then added a fourfold volume of ethyl acetate to the acidified supernatants to extract RhL into the ethyl acetate phase. One ml of the ethyl acetate extract was then taken and evaporated at 95 °C. Then, the common anthrone analysis was done to determine the RhL concentration, as described in the previous report.

To correlate cell dry weight (CDW) and intracellular protein (IP) concentration 2 mL samples at different stages of fermentation were taken: one sample each from the two growth phases and the stationary phase. We centrifuged the sample, removed the supernatant, resuspended the cell pellet in deionized water, centrifuged again to remove the wash solution, resuspended the washed cells in deionized water, poured the cell suspension to a pre-weighed weighing pan, dried the cell suspension in an oven set at 105 °C overnight, and measured the cell dry weight. In parallel, we analyzed the same samples using the standard Bradford method to determine their intracellular protein concentrations. **Figure 2** shows the correlation between CDW and IP from these samples, suggesting that protein makes up about 45% of cell dry weight.

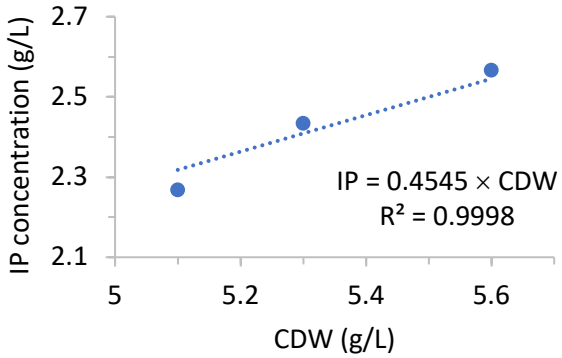

Figure 2: Intracellular protein concentration vs. cell-dry-weight concentration in fermentation samples

Milestone 2, Corrosion (PW simulant).

In the FY 24 Q1-3 reports, we described the procedure for evaluating RhL efficiency in a produced water simulant, specifically, corrosion current density measurements from potentiodynamic polarization curves for C1018 carbon steel in a rotating cylinder electrode setup (1000 rpm) in a 1% NaCl solution saturated with CO_2. In addition, we reported that %IE was on the order of 98% across the concentration range of 10 ppm to 200 ppm.

In this quarter, we used linear polarization resistance combined with mass loss and electrochemical impedance spectroscopy to monitor corrosion rate over a 14 day period. As before, C1018 carbon steel in a RCE setup (1000 rpm) in a 1% NaCl solution, 100 ppm RhL and saturated with CO_2 was used. In these measurements, the specimen was allowed to remain at the open circuit potential (OCP) and, every 12 hours, it was polarized a small amount about its OCP (+/- 20 mV). As the polarization is small, the method is considered non-destructive (unlike the cathodic polarization curves used to measure %IE). Near the OCP, the current - potential response of the specimen is linear and, as such, the slope of this plot yields a resistance, the polarization resistance. A typical plot is shown in **Figure 3** from the RCE experiment.

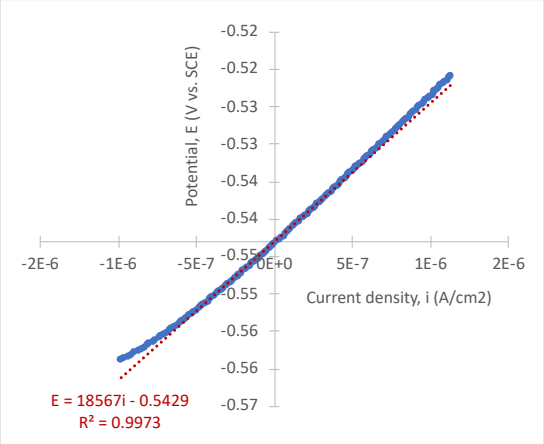

Figure 3 Typical LPR data for C1018 carbon steel in a rotating cylinder electrode setup (1000 rpm) in a 1% NaCl solution, 100 ppm RhL and saturated with CO_2 was used. The slope of the curve near the OCP (-0.543 V SCE) is equal to the polarization resistance, Rp. Data taken upon the initial exposure in the RCE set-up (t = 0).

A summary of Rp as a function of exposure time is presented in **Figure 4**. As can be seen in this figure, the value of Rp increased by a factor of more than 2 over the 14 day exposure. Higher Rp correlates with lower corrosion rates as discussed below. In addition, Rp increased with increasing E_{corr} as might be expected for a passivated surface. The origin of the step in Rp at approximately 80 hrs is not clear and we are in the process of duplicating these results.

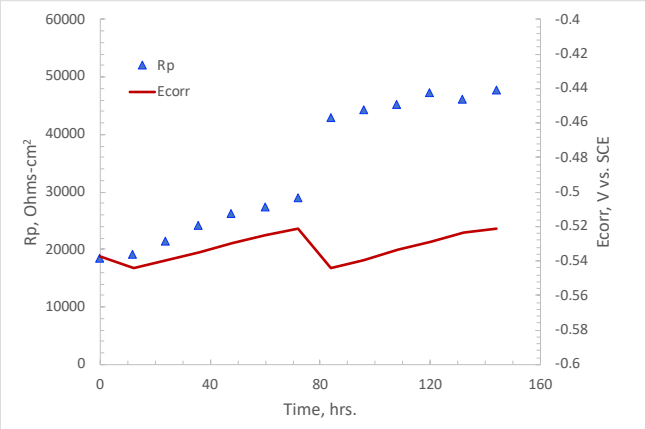

Figure 4 Summary of polarization resistance results as a function of exposure time for for C1018 carbon steel in a rotating cylinder electrode setup (1000 rpm) in a 1% NaCl solution, 100 ppm RhL and saturated with CO_2.

Corrosion current density and, correspondingly, corrosion rate can be calculated from polarization resistance in **Figure 4**. From Ohm's law, R=V/I., the polarization resistance, R_p is defined as:

$$R_p = \frac{\beta_a \beta_c}{2.303 i_{corr}(\beta_c - \beta_a)} \quad . \tag{1}$$

In **Eq. 1**, β_a and β_c are the Tafel slope (in volts) as measured in the potentiodynamic polarization data shown in previous reports. Commonly, **Eq. 1** is written as:

$$i_{corr} = \frac{B}{2.303 R_p} \quad , \tag{2}$$

where:

$$B = \frac{\beta_a \beta_c}{(\beta_c - \beta_a)} \quad . \tag{3}$$

Calculations of i_{corr} from mass loss data measured after the LPR experiment are compared to i_{corr} from LPR measurements in **Table 2**. As seen in this table, corrosion rate in the inhibited solution is low, less than 1 mpy. In comparison, corrosion rates in the uninhibited solution are on the order of 60 mpy, i_{corr}=1.26x10^{-4} A/cm^2 (see previous reports).

Table 2 Calculations of corrosion rate from LPR and mass loss.

Constants		**Mass Loss Data**		**LPR Data**	
area, cm^2	3.0	mass, g, start	5.2063	avrg Rp, Ω-cm^2	33839
β_a, V	0.056	mass, g, end	5.2055		
β_c, V	-0.249	mass loss, g	0.0008		
EW g/mol e-	27.92365	exposure time, s	6048000		
F, C/mol e-	96485				
density, g/cm^3	7.86	**i_{corr}, A/cm^2**	1.52x10^{-6}	**i_{corr}, A/cm^2**	9.27x10^{-7}
		CR, mpy	0.70	**CR, mpy**	0.428

The final work in the PW simulant will be to learn more about the mechanism of RhL inhibition and solution properties that effect it. We mentioned above the use of EIS as one method for doing this and the results from those experiments to be presented later. Another method for

investigating solution properties that influence inhibition is the quartz crystal microbalance (QCM). The QCM measures adsorbed mass by measuring the change in resonant frequency of a coated quartz crystal (here Au on Ti). One of the first things we are measuring in these studies is RhL adsorption as a function of pH. In these experiments two buffer solutions were used, phosphate buffer with a pH of 8 and an acetic acid buffer with a pH of 4. pH was the first solution property investigated as it effect RhL micelle formation. Preliminary results from these experiments are presented in **Figure 5** where the graph plots change in resonance frequency with time. In these experiments, the baseline resonance frequency in the buffer solution was recorded for a period of time prior to the addition of RhL. Once RhL was added (approx. 1000 s pH 4, 2000 pH 8) a change (decrease) in resonance frequency was observed consistent with an increase in crystal mass. Typically, this change in mass is calculated using the Saurbrey equation, however, in this case, the decrease in resonance frequency observed in **Figure 5** owes both to the mass associated with physical adsorption as well as interaction of adsorbed RhL with RhL in solution, e.g. a viscoelastic effect. This will be further addressed in future investigations and a rigorous analysis of the data in **Figure 5** will be presented.

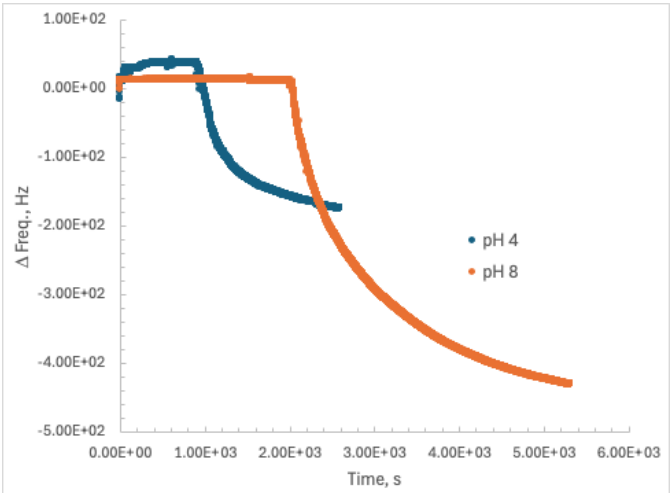

Figure 5 QCM results showing adsorption of RhL (100 ppm) to a gold surface as a function of pH.

Milestone 3, SRB-MIC.

Desulfovibrio vulgaris (American Type Culture Collection 7757) will be cultured in anaerobic Postgate medium C. One liter of this medium contains 6 g sodium pyruvate (C_3H_3NaOs), 4.5 g sodium sulfate (Na_2SO_4), 1 g yeast extract, 1 g ammonium chloride (NH_4Cl), 0.5 g monopotassium phosphate (KH_2PO,), 0.3 g sodium citrate dihydrate ($Na_3C_3H_3O_7$-$2H_20$), 0.06 g magnesium sulfate heptahydrate ($MgSO_4$-$7H_2O$), 0.04 g calcium chloride hexahydrate ($CaCl_2$-$6H_20$), 0.004 g ferrous sulfate heptahydrate ($FeSO_3$-$7H_20$) and a cysteine stock solution to achieve a final concentration of 0.002 g/l. The pH is adjusted to 7.0 by addition of 1 M NaOH solution.

Prior to culturing the bacteria and conducting the SRB-MIC tests, C1018 corrosion rate in Postage C with and without RhL was measured using mass loss to establish a baseline for SRB tests. In these experiments, C1018 specimens were ground to 600 grit SiC and cleaned using acetone/ethanol/DI water in an ultrasonic cleaner. The mass of the specimens was then recorded. Postgate C was then prepared in the serum bottles used for the exposure experiments and

subsequently sterilized, along with all other glassware by autoclaving at 121 °C for 20 min. Argon was bubbled through the medium overnight (12 hr. min.) to remove O_2 prior to inserting the C1018 specimens. In this procedure a 0.2 μm filter was used to sterilize the Ar gas. The C1018 steel specimens, attached to bottle caps specifically designed for these types of exposure experiments, were transferred to the media bottles in a glove bag that was also purged with Ar. We anticipate a 30 day exposure period for these experiments and the results will be reported in the FY '25 Q2 report.

Project Financial Activities Incurred during the Reporting Period:
Since the beginning of the project, we have spent $136,858.45 (with encumbrances): $68,347.56 salary, $14,065.10 fringe, $10,974.21 supplies $46,820.00 indirect cost. The spending break down is shown below.

Ledger Account	Budget	LTD Actuals	Total Spend (with Encumbrances)	Remaining Balance	% Remaining
Salary	$234,584.000	$68,347.560	$68,347.560	$166,236.440	70.86%
5000:Full Time Faculty	$22,567.000	$22,566.580	$22,566.580	$0.420	0.00%
5040:Summer Faculty	$51,289.000	$36,371.000	$36,371.000	$14,918.000	29.09%
5300:Graduate Assistants	$136,000.000	$6,923.160	$6,923.160	$129,076.840	94.91%
5400:Student Assistants	$24,728.000	$2,486.820	$2,486.820	$22,241.180	89.94%
Fringe Benefits	$20,599.000	$14,065.100	$14,065.100	$6,533.900	31.72%
Supplies & Services	$18,600.000	$7,625.790	$7,625.790	$10,974.210	59.00%
Student Aid	$30,000.000	$0.000	$0.000	$30,000.000	100.00%
7000:Travel	$10,000.000	$0.000	$0.000	$10,000.000	100.00%
Total Direct Costs	$313,783.000	$90,038.450	$90,038.450	$223,744.550	71.31%
Indirect Cost	$147,567.000	$46,820.000	$46,820.000	$100,747.000	68.27%
Total Direct & Indirect Costs	$461,350.000	$136,858.450	$136,858.450	$324,491.550	70.34%

Project Activities with Cost Share Partners:
None.

Project Activities with External Partners:
None.

Potential Project Risks:
Our co-investigator at BP, Tim Bieri, who retired on Dec. 33 2024 will be replaced at BP by Shokrollah Hassani. We are in the process of setting up a meeting with Mr. Hassani to discuss with him our goals, our anticipated crude specimen needs as well as to update him on our progress to date. While this may have a small impact on our timeline for obtaining specimens we see no major disruption.

Future Project Work:
During Q2 of FY ’25, the PIs anticipate working on the following topics:

1) Reviving and expanding of *D. vulgaris* in Postgate C purged by Ar; designing the flow system to follow the attachment study of *D. vulgaris* on C1018 coupons and the corrosion of C1018 by *D. vulgaris*; alternatively, if the flow system is proven to be un-feasible for following attachment under sterilized anaerobic conditions, a semi-flow system using serum bottle with periodic republishing of fresh Postgate C will be adopted. The C1018 steel specimens will be attached to bottle caps and exposed to the *D. Vulgaris* in Postgate

C. The C1018 specimen will be periodically removed to exam biofilm formation and corrosion extent.

2) Confirming *P. aeruginosa* seed culture purity and, potentially, screening colonies for RhL productivity. The culture was previously isolated and selected from soil samples near a biodiesel production plant. The strain had been maintained in the laboratory for about 10 years. Performing the colony rescreening again may be helpful to ensure maximal RhL productivity.
3) Identifying the limiting non-C, non-N nutrient in the RhL fermentation. Optimal RhL production relies on maintaining an active RhL-producing but non-growing bacterial culture, with the growth being limited by a non-C, non-N nutrient. In our previous work, we did not determine which nutrient was actually limiting the cell growth. We have planned a series of shake flask experiments to determine the limiting nutrient(s). With that knowledge, we hope to be able to further improve the RhL productivity by adjusting the mineral nutrient concentrations.
4) Corrosion rate measurements and %IE in a crude simulant. The crude simulant will contain both an aqueous phase and an organic phase. We anticipate the main effect of an organic phase will be on the partitioning of RhL between this phase and the water phase (PW simulant), e.g. the presence of the organic phase will lower RhL concentration in aqueous phase (where the inhibitor is needed). The first step in this process will be to measure the concentration of RhL in the aqueous phase for a 2-phase simulant. For the aqueous phase we will continue to use the PW simulant described above and the composition of the organic phase will be discussed in the ’25 Q2 report. The second step will be to measure corrosion rate measurements and %IE in the crude simulant.
5) Static mass loss SRB experiments. Our SRB /MIC investigation has two goals: effect of RhL on inhibition of SRB attachment and effect of RhL on MIC mitigation. In this quarter we began examining the ability of RhL to decrease corrosion rates in an SRB environment. In these experiments we are measuring mass loss as a function of RhL concentration and we anticipate reporting preliminary results in Q2 and final results in Q3. In Q2 we will begin our investigation of SRB attachment and morbidity as a function of RhL concentration. We anticipate the attachment and morbidity will correlate directly with corrosion rate measurements.

Potential Impacts to Pipeline Safety:

FY ’25, Q1 – The results, thus far, are extremely encouraging. We have verified our initial experiments that show RhL may be an effective inhibitor crude pipelines.